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The major objective of this research project is to identify and characterize promoter/enhancer elements that can be used to specifically target the expression of therapeutic genes to hypoxic regions within prostatic tumors *in vivo*. We have characterized sequences present within the human TNF-α promoter that are responsible for both constitutive activity and induction by hypoxia. We have produced a mutated version of this promoter that exhibits substantially reduced constitutive activity in epithelial cells while retaining responsiveness to various activating stimuli including hypoxia and ionizing radiation. While lacking prostate specificity, it is hoped nevertheless that this element may prove useful in various cancer gene therapy applications. In addition, we have cloned and characterized a large panel of novel sequences that exhibit high levels of constitutive promoter activity in prostate cell lines. Finally, we have identified a number of hypoxia inducible-promoter element and are currently characterizing these with respect to their prostate specificity.

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INTRODUCTION

The major objective of this research project is to identify and characterize promoter/enhancer elements that can be used to specifically target the expression of therapeutic genes to hypoxic regions within prostatic tumors *in vivo*. Specifically, a novel episomal promoter/enhancer trap cloning system developed in our Laboratory is being used to rapidly isolate candidate DNA sequences that are then tested both *in vitro* and *in vivo* for their functional activity in a variety of prostatic and other tumor cell lines under both normoxic and hypoxic conditions. Ultimately, adenoviral vectors in which expression of an indicator gene is driven off a candidate promoter/enhancer element will be constructed and tested for their ability to specifically target gene expression to hypoxic regions of prostatic tumor xenografts *in vivo*.

BODY/PROGRESS (YEAR 1)

Task 1: Isolation and characterization of novel "prostate-specific" hypoxia inducible promoter/enhancer elements (months 1-12)

In order to directly isolate novel hypoxia-inducible promoter/enhancer elements for use in prostatic cancer gene therapy, we have developed a unique episomal promoter/enhancer expression cloning strategy based on a vector that we have generated designated pEGTIII. This vector contains a "leaderless" bone/liver/kidney alkaline phosphatase cDNA (ALP) that has been engineered to express a widely utilized splice acceptor site derived from CD44 exon v9 at its 5' end. A library of randomly-generated size-selected (mean size around 2.5 kb) DNA fragments were cloned upstream of this indicator gene. Upon transfection into an appropriate eukaryotic target cell (i.e. DU145), expression of ALP will only occur if the DNA fragment present in the plasmid vector contains both an appropriate promoter/enhancer element and an exon that can provide a splice donor site that can be utilized to generate an in-frame fusion protein. The vector also contains both a gene encoding EBNA-1 and the EBV origin of replication (oriP) and as such replicates episomally within the nucleus of most human cell types giving approximately 20-30 copies/cell. Such amplification is an important component of the cloning strategy as it may help exclude undesirable promoter elements with weak constitutive activity, thereby favoring the isolation of promoters that are inactive in prostatic cells under normoxic conditions.

In practice, the plasmid library was introduced into prostatic tumor cell lines by electroporation. Forty-eight hours later, G418 was added at a final concentration of 200-400 μg/ml to select for transfected cells. Plates were then cultured for 18-21 days during which time discrete colonies containing 100-200 cells are produced. We have developed a colony lift technique in which plates are briefly overlaid with Immobilon-P PVDF membranes. Following fixation in methanol the membranes are stained with the ALP substrate BCIP/INT. Colonies that produce ALP, presumably because they were transfected with a plasmid containing a promoter/enhancer element that is constitutively active in the cell type under study, can be readily identified by the presence on the membrane

of a brown staining "plaque". The position of such colonies on the original plate is marked, fresh media added and the cells cultured for a further 48 hours to allow recovery. At this point, plates are transferred to a tri-gas incubator containing a defined hypoxic atmosphere composed of 0.5% O_2, 5% CO_2 and 94.5% N_2. Eighteen hours later, tumor cell colonies are fixed in 100% ethanol for 1 minute and then stained with the ALP substrate BCIP/INT. Colonies in which ALP expression was induced by exposure to the hypoxic microenvironment (because they contain a promoter/enhancer element that responds to this stimulus) can be readily identified and differentiated from the previously marked colonies that contain constitutively active promoters. Plasmid DNA can be recovered from positively staining colonies by scraping the cells from the surface of the dish, performing a Hirt extraction and then transforming the DNA obtained into a competent bacterial host. Individual bacterial colonies are picked, plasmid DNA once again isolated and transfected back into the tumor cell line which is then stimulated as before in order to ensure that a particular clonal plasmid does indeed contain a promoter/enhancer element that is responsive to hypoxia.

Confirming the validity of this unique experimental approach, a 1399 bp genomic DNA fragment corresponding to a region of the human TNF-α promoter (position -1307 to +92) has been isolated, cloned into a modified version of the pEGTIII vector containing a full-length ALP cDNA (pEGTIV) and shown to possess both low constitutive activity and good induction following 18 hour incubation of transfected K562 cells (erythroleukemia) in a low O_2 environment. Unfortunately, in prostatic and other epithelial cell lines, although significant induction was also obtained following exposure to hypoxia and/or other stimuli, the TNF-α promoter exhibited high constitutive levels of activity that severely limit its usefulness in targeted gene therapy settings. Using extensive and systematic deletional analysis it was determined that a sequence element present within a ~100 bp region immediately upstream of the TATA box was responsible for the majority of this background activity. Individual transcription factor binding sites located within this region were then targeted using a PCR-based site-directed mutagenesis approach and in this way, the AP1 site located at position -108 was shown to play a critical role in the determining the constitutive activity of the promoter in non-myeloid cells. When placed upstream of a GFP indicator gene a "full length" TNF-α promoter in which this site was rendered nonfunctional exhibited minimal background activity but retained responsiveness to various stimuli including hypoxia and ionizing radiation. Using this construct as a starting point, additional deletional analysis was carried out to define the sequence motif(s) responsible for the observed hypoxia-induced activity of the TNF-α promoter. Particular emphasis was placed on the three NF-κB sites shared by both the human and mouse TNF-α genes as changes in redox potential resulting from irradiation or exposure to hypoxic conditions induce activation of NF-κB and tyrosine phosphorylation of its inhibitory subunit IκBα via a signal transduction pathway that involves Ras and Raf, but not MAP kinase. Moreover, recent studies have shown that interaction between NF-κB and the κ3 site located toward the 3' end of the promoter plays an important role in the transcriptional activation of TNF-α by superantigen. In contrast, studies with the monocytic cell line Mono Mac 6 indicate that LPS induces a factor with the

characteristics of NF-κB that interacts with κ1, the most 5' of the three NF-κB binding sites in the promoter. Although definitive site-directed mutagenesis studies have yet to be completed, characterization of the deletional mutants generated in the present study suggest that it is the κ1 NF-κB motif that is largely responsible for the hypoxia-inducible activity of the element.

The pEGTIII vector and derivatives thereof have also been used to isolate a large number of defined promoter elements that exhibit high levels of constitutive activity in prostatic tumor cell lines. Systematic sequencing of these novel elements is currently underway. Among the most promising with respect to restricted cellular specificity is a sequence of approximately 2 kb corresponding to a region of the human X chromosome (position Xp21.1). In order to further narrow down functionally important regions within this and other constitutively active promoter elements, a series of deletion mutants are being generated using standard restriction enzyme digestion, PCR and/or exonuclease treatment (as was used in the characterization of the TNF-α promoter) and the activity and specificity of the mutants produced again tested following transfection into a panel of prostatic and other tumor cell lines. Ultimately, site-directed mutagenesis will be used to precisely identify the transcription factor binding sites responsible for the constitutive activity of candidate promoters in prostatic tumor cells.

Finally, a number of promising hypoxia-inducible elements have also been isolated using the experimental approach described above. These are in the process of being cloned into the pEGTIV vector upstream of a full length ALP cDNA, and their activity, cellular specificity and hypoxia responsiveness will once again tested in a variety of prostatic and other tumor cell lines under both normoxic and hypoxic conditions.

Task 2: Characterization of the functional activity of candidate promoter/enhancer elements in prostatic tumor cells *in vitro* and *in vivo* (months 7-18)

The major objective of this task is to characterize the functional activity of candidate hypoxia-inducible promoter elements in tumor spheroids that contain regions of diffusion-limited hypoxia. Proof of concept studies using DU145 cells transfected with pEGTIV vectors in which ALP indicator gene expression is driven off either constitutively active promoter elements or the various human TNF-α promoter mutants described above, are currently underway. Promising hypoxia-responsive elements will be similarly characterized in due course.

Task 3: Generation and analysis of adenoviral vectors in which expression of an indicator gene is driven off candidate hypoxia-inducible, prostate-specific promoter elements (months 18-30)

In anticipation of initiating this important phase of the project, control adenoviral vectors have been generated in which expression of various indicator genes is driven off a number of constitutive promoter elements. Rather than using the approach outlined in the initial proposal, these were constructed using the

AdenoQuest Kit sold by Quantum Biotech Ltd., as the design of the "transfer" vector included in this kit facilitates the direct subcloning from pEGTIV of a cassette that includes the promoter of interest, the ALP indicator gene and an SV40-derived polyadenylation signal. The adenoviral vectors that have been generated to date will serve as important controls against which the activity of novel hypoxia-inducible promoter elements will be measured following direct injection into solid tumour masses. They have also been used in initial studies to confirm that prostatic tumors can be efficiently transduced *in vivo* using such vectors.

KEY RESEARCH ACCOMPLISHMENTS:

- Identification of the transcription factor binding sites present within the human TNF-α promoter responsible for constitutive activity and hypoxia responsiveness in transfected prostatic tumor cells.
- Isolation and preliminary characterization of a panel of promoter elements that exhibit high levels of constitutive activity in prostatic tumor cells.
- Isolation and preliminary characterization of candidate prostate-specific hypoxia-responsive promoter elements.

REPORTABLE OUTCOMES:

It is our intention within the next few months to submit a manuscript describing the characterization of the transcription factor binding sites present within the human TNF-α promoter that are responsible for constitutive activity and hypoxia responsiveness.

The sequences of the novel promoter elements identified in this study will be submitted to GENBANK once these have been confirmed.

Aliquots of the pEGTIII library have been provided to various colleagues in the US and abroad who are interested in identifying promoter elements that respond to a variety of stimuli including, hypoxia, radiation and tubulin-binding agents.

CONCLUSIONS:

This study has progressed very much to plan. We have characterized the sequences present within the human TNF-α promoter that are responsible for both constitutive activity and responsiveness to hypoxia. We have produced a mutated version of this promoter that exhibits substantially reduced constitutive activity in epithelial cells while retaining responsiveness to various activating stimuli including hypoxia and ionizing radiation. While lacking prostate specificity, it is hoped nevertheless that this element may prove useful in various cancer gene therapy applications. In addition, we have cloned and characterized a large panel of novel sequences that exhibit high levels of constitutive promoter activity in prostate cell lines. Finally, we have identified a

number of hypoxia inducible-promoter elements which are currently being characterized with respect to their prostate specificity.

REFERENCES:

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